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## Effect of S-(Dichlorovinyl)-L-Cysteine on Incorporation of Precursors into Deoxypentose Nucleic Acid of Leukocytes.* (25601)

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Continuing our investigations on the mechanism by which trichloroethylene-extracted soybean oil meal or S-(dichlorovinyl)-L-cysteine (DCVC)(1) can induce fatal aplastic anemia in calves we studied the effects of DCVC on incorporation of radioactively labeled precursors into deoxypentose nucleic acid (DNA) of leukocytes.

*Procedures. Experiments with $P^{32}$-phosphate.* Male Holstein calves, weighing 100-130 lb were conditioned at least 2 weeks prior to experiments until judged normal by clinical and hematological observations. Sodium $P^{32}$-phosphate, (2.5 mc, specific activity: 48-208 c/g, pH = 7.4, in 0.9% aqueous sodium chloride) was injected intravenously. A dose of DCVC (total of 3.3 mg/kg in 3 equal portions during 10 hr), previously found to induce fatal aplastic anemia in young calves(1), was injected intravenously on same day as $P^{32}$ (first portion 5 hr before $P^{32}$; 2 calves) or 2 days prior to $P^{32}$ (4 calves), or 4 days prior to $P^{32}$ (2 calves). Four calves receiving no DCVC served as controls. All calves were kept in metabolism cages and fed, twice daily, suspension of hexane-extracted soybean oil meal in milk. Counts of thrombocytes, total and differential leukocytes were made daily. Heparinized blood (75 ml) was collected 6 hours after $P^{32}$ injection and then daily. It was centrifuged at 5°C and 2000 g for 30 minutes, plasma removed and recentrifuged. One ml of plasma and 0.1 ml of glycerol were placed in aluminum planchets lined with filter paper and dried under infrared lamp for measurement of radioactivity. The buffy coat was lifted out, briefly washed with cold 0.16 M sodium chloride-0.01 M sodium citrate solution and DNA isolated by the method of Kay *et al.*(2). DNA-containing precipitate was suspended in 2.5 ml of 5% trichloroacetic acid, heated at 100°C for 30 minutes, cooled, diluted to 5 ml with $H_2O$, and centrifuged. Aliquots of clear supernatant were dried on paperlined planchets to measure radioactivity, or used for DNA determinations(3). Specific activity of leukocyte DNA was calculated as counts/minute (CPM)/$\mu$g DNA. The collected urine was filtered, 1 ml aliquots dried in paperlined planchets under infrared lamp. Radioactivity was measured with gas-flow, thin window Geiger counter and calculated with corrections for physical decay and self-absorption.

Isolated leukocyte DNA was checked for contamination by RNA and by inorganic $P^{32}$. RNA was determined in trichloroacetic acid extracts by the orcinol method(4). To check for inorganic $P^{32}$ contamination, $P^{32}$-phosphate was added to chilled buffy coat suspension at concentrations comparable to those found in plasma, and isolation procedure for DNA was initiated at once. Radioactivity of isolated DNA was then determined. *With tritiated thymidine.* Incorporation of this compound into leukocyte DNA was studied *in vitro* with blood from normal and DCVC-treated calves. Fresh blood containing 1/10 volume disodium ethylenediamine tetraacetic

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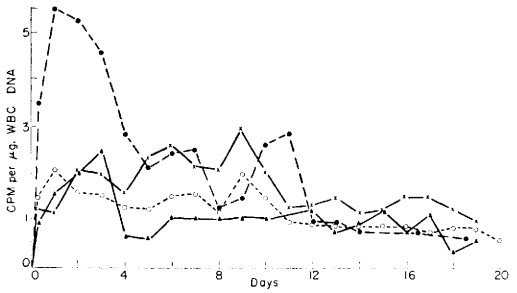


FIG. 1. Specific activity of leukocyte DNA after inj. of $P^{32}$-phosphate and DCVC. ●----● no DCVC (means of 4); ×——× DCVC on same day as $P^{32}$ (means of 2); ○- - -○ DCVC 2 days before $P^{32}$ (means of 4); ▲——▲ DCVC 4 days before $P^{32}$ (means of 2).

acid (1% in 0.7% aqueous sodium chloride) was placed in silicone-coated tubes, and $H^3$-thymidine† (specific activity: 360 mc/m mole) was added to final concentration of 5 µc/ml. This mixture was kept at room temperature and smears were made after 1 and 6 hours. Effects of DCVC on $H^3$-thymidine uptake were also studied *in vitro* by addition of DCVC to normal calf blood at concentration (1 µM/ml) which retards growth of cells in tissue culture.‡ Autoradiographs were made with Kodak Autoradiographic Stripping Film AR-10, exposed for 6 days at 4°C, developed with Kodak D19b, fixed with Kodak acid fixer, washed and stained with Leishmann-Giemsa stain(5).

*Results.* Control calves had always normal blood counts. In blood of DCVC-treated calves, as observed previously(1), counts of leukocytes, thrombocytes and polymorphonuclear leukocytes remained normal for at least 12-14 days, then progressively dropped to very low levels. The DCVC-treated calves died after 20-30 days.

Fig. 1 shows that treatment of calves with lethal dose of DCVC decreased but did not abolish early incorporation of inorganic phosphate into DNA of blood leukocytes. With normal calves a maximum specific activity of about 2½ times that found with DCVC-treated calves was observed 1-2 days after administration of $P^{32}$. In normal calves there occurred a second, distinct rise in specific activity of leukocyte DNA between 9th and 11th day when concentration of $P^{32}$ in blood plasma had greatly decreased, essentially to same extent as in DCVC-treated calves. No evidence was found for this in calves treated with DCVC 4 days before administration of $P^{32}$. Leukocyte DNA was obtained from a mixed population of cells in which lymphocytes predominated(1); no information is available on rate of DNA synthesis in different cell types or their precursors in the calf.

Radioactivity of DNA extracts was not the result of contamination with inorganic phosphate. When $P^{32}$-phosphate was added to a suspension of buffy coat from normal calves and DNA isolation started immediately, radioactivity recovered in DNA extract was 0.12% of the added $P^{32}$. Actual contamination of DNA with inorganic $P^{32}$ in *in vivo* experiments is probably less than this; however it is not known at what stage of isolation procedure incorporation of $P^{32}$ into leukocyte DNA stops completely. When DNA extracts were treated with orcinol reagent, colored products were obtained which, when calculated as pentose nucleic acid (PNA), gave results indicating that PNA was only about 1% of DNA present. Inasmuch as authentic DNA also gives an orcinol reaction of this order of magnitude(4), it is assumed that PNA contamination of DNA extracts was negligible.

Calves treated with DCVC 2 or 4 days before injection with $P^{32}$, had during the first 2 days a higher rate of urinary $P^{32}$ excretion. This may reflect some kidney damage which becomes severe and results in high blood urea nitrogen values when higher doses (4-5 mg/kg) of DCVC are injected.‡ In these experiments blood urea nitrogen remained normal.

When blood from normal calves was incubated with $H^3$-thymidine, about 1% of mononuclear cells showed nuclear uptake of the tracer, presumably into the DNA. No uptake of radioactivity was observed in polymorphonuclear leukocytes. Appreciable labeling took place in 1 hour, and corroborated the *in vivo* studies in which rapid incorporation of $P^{32}$ into blood leukocyte DNA was observed. DCVC did not inhibit incorporation of $H^3$-thymidine into these cells, when collected from DCVC-treated calves (Fig. 2A) or when nor-

† Purchased from Schwarz Labs., Mount Vernon, N. Y.

‡ Our unpublished observations.

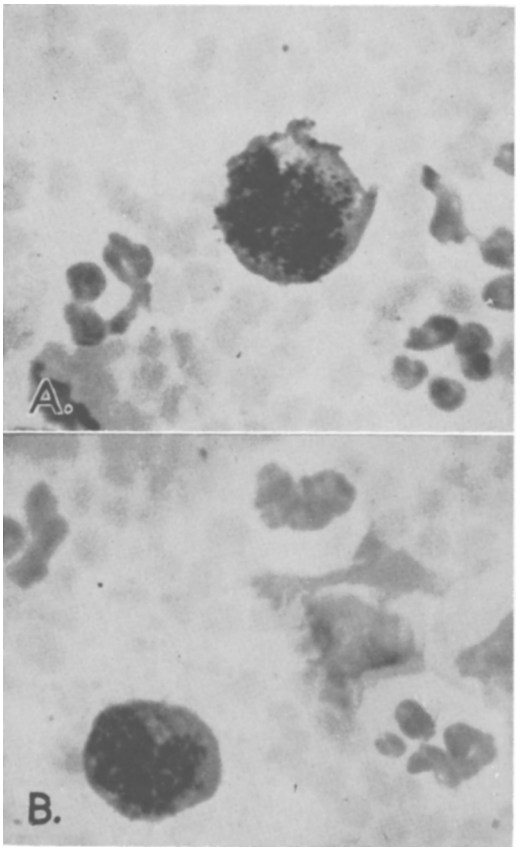


FIG. 2. Radioautographs of blood treated *in vitro* with tritiated thymidine. ×1200. A. Blood from calf treated with DCVC 18 days before collection of blood. B. Blood from normal calf incubated with DCVC (1 μmole/ml, added at same time as thymidine).

mal blood cells were incubated *in vitro* with DCVC in presence of $H^3$-thymidine (Fig. 2B).

*Discussion.* Many studies have been made with radioactively-labeled blood cells, but problems of dynamics of leukocyte production and destruction remain to be resolved(6). They are complicated through the presence of mixed populations of cells, formation of each probably being subject to independent controls. Moreover, there is evidence that leukocytes in circulation may represent only a small fraction of total leukocytes in the body, and there is little information on size and location of leukocyte pools, or on rate and extent of exchange of blood leukocytes with those in extravascular pools. Of tracer methods employed, labeling of DNA offers advantages because isotope incorporation occurs only with concurrent DNA synthesis in cells preparing for cell division, and because the label remains stably bound to DNA during lifetime of the cell; hence cells with labeled DNA represent newly formed cells. Analysis of $P^{32}$ concentration in leukocyte DNA has been made in man(7-12), dog(13,14) and rabbit (15,16). Changes in specific activity observed in normal calves (Fig. 1) resemble the biphasic curves observed with rabbits(15,16). The fact that lymphocytes predominate in both bovine and rabbit blood may be relevant. In present studies, radioautograms were made of blood smears from $P^{32}$-treated calves, but the level of radioactivity was too low for satisfactory results.

Control calves showed no evidence of radiation damage, from examination of peripheral blood levels of hemoglobin, thrombocyte, leukocyte or differential counts after treatment with 2.5 mc $P^{32}$. This amount is less than 5 mc used for treatment of polycythemic human subjects of similar body weight(17).

When blood was incubated with $H^3$-thymidine, about 1% of mononuclear leukocytes showed intense labeling after 1 hour. The number of these cells did not increase appreciably after 6 hours' incubation. The majority of labeled cells were large, and resembled the mononuclear cells which Bond, Cronkite and their associates(18) suggested to be possible multipotential primitive cells with proliferative capacity, in transit in blood. Early uptake of $P^{32}$ into DNA could be explained by presence of these leukocytes, capable of DNA synthesis while in circulation, therefore no delay would occur in appearance of labeled cells, in contrast to granulocytes(6). Either with normal blood to which DCVC had been added or with blood from DCVC-treated calves, the nuclear uptake of $H^3$-thymidine into these mononuclear cells was not inhibited.

*Summary.* 1. When sodium $P^{32}$-phosphate was injected intravenously into calves, there was an early labeling of leukocyte DNA. 2. DCVC decreased but did not abolish this uptake of $P^{32}$ when given concurrently with $P^{32}$ or when calves were pre-treated with DCVC for 2 or 4 days. 3. When normal calf blood was incubated in presence of $H^3$-thymidine,

there was a rapid nuclear uptake by mononuclear leukocytes. 4. DCVC did not inhibit incorporation of $H^3$-thymidine in either blood from DCVC-treated calves or in normal blood incubated in presence of DCVC. 5. Abolishment of DNA synthesis in leukocytes or leukopoietic tissue does not appear to be a primary biochemical lesion through which DCVC induces fatal blood dyscrasia in calves.

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## Analgesia Enhancing Effect of Thalidomide. (25602)

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Since it has been reported that addition of thalidomide* (N-phthalyl-glutamic acid imide) to the classical "APC" combination enhanced its clinical analgesic effect(1), it was considered interesting to study this effect experimentally on human subjects.

*Method.* The amount of electricity necessary to elicit a painful sensation was determined 3 times at 15 minute intervals before, and 9 times at 20 minutes after oral medication. The current was applied as square waves of 1 msec. duration every half second. The stimulus was increased by 0.01 volt at every impulse until the subjects indicated feeling pain. Temporal clues during this procedure were avoided. The method has been described(2,3). Each of the 10 subjects served for 5 experimental sessions, with at least 48 hours intervening between any 2. The sequence of medication was by "latin-square" design and "double-blind" discipline prevailed. The medications, prepared to look alike were: A. 2 APC, = 7 gr. acetylsalicylic acid, 5 gr. acetophenetidin, 1 gr. caffeine; B. 25 mg thalidomide + 2 APC; C. Placebo; D. 32 mg Codeine sulfate + 2 APC; E. "Dry-run" (no capsules).

*Results.* For analysis, each postmedication threshold was expressed as its increment from its average premedication voltage on the same day. The average threshold increments of the 10 subjects, by interval after and type of treatment, are presented in Table I.

An analysis of variance, performed on the detailed aggregated data (of which Table I is a summary), is summarized in Table II. This analysis compares the net (or average or areas under the curve) increments following each treatment, in terms of variance attributable to subject-treatment interaction. No significant difference was found between the average

* "Kevadon", Trademark of Wm. S. Merrell Co.